Journal of Medical Virology 57:174–178 (1999)

IgG Immune Response to B19 Parvovirus VP1 and VP2 Linear Epitopes by Immunoblot Assay

Elisabetta Manaresi, Giorgio Gallinella, Marialuisa Zerbini, Simona Venturoli, Giovanna Gentilomi, and Monica Musiani*

Department of Clinical and Experimental Medicine, Division of Microbiology, University of Bologna, Bologna, Italy

Human B19 parvovirus recombinant capsid proteins VP1 and VP2 were expressed in *E. coli* and purified. Recombinant proteins were used to detect a specific IgG immune response against VP1 and VP2 linear epitopes by immunoblot assay. A total of 222 serum samples from 218 apparently immunocompetent subjects with different clinical conditions and laboratory evaluations with regards to B19 infection were analyzed. The sera had previously been tested for B19 DNA and for specific IgM and IgG against VP2 conformational antigens by ELISA assay. The data show that, during the active or very recent phase of infection, IgG anti-VP1 linear epitopes appear in concomitance and with the same frequency as IgG anti-VP2 conformational antigens. IgG against conformational VP2 antigens and against linear VP1 epitopes seem to persist for months or years in the majority of individuals. IgG against VP2 linear epitopes are generally present during the active or very recent phase of infection and during the convalescent phase, while they are present only in about 20% of subjects with signs of a past B19 infection. ***J. Med. Virol. 57:174–178, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: B19 immune response; recombinant proteins; Western blot; linear antigens

INTRODUCTION

Human parvovirus B19 is the etiological agent of a wide range of clinical syndromes such as erythema infectiosum, fetal hydrops, postinfectious arthropathy, transient aplastic crises in patients with hemolytic disorders, and chronic bone marrow failure in immunocompromised patients [Anderson, 1990; Brown et al., 1994; Pattison, 1994]. As B19 parvovirus cannot be efficiently grown in established cell cultures, the diagnosis of B19 infection mainly relies on the detection of specific immune response to B19 and/or on the direct detection of B19 genomes or antigens.

Serological procedures to detect antibodies against the two capsid proteins, VP1 and VP2, which represent about 5% and 95% of the B19 capsid, respectively, are available tools to diagnose recent B19 infections and also to determine the immune status against the virus.

Serological tests generally use as source of antigens recombinant B19 proteins that have been expressed in several systems, both prokaryotic and eukaryotic [Morinet et al., 1989; Brown et al., 1990; Kajigaya et al., 1991; Söderlund et al., 1992]. B19 structural proteins, expressed in eukaryotic systems, can self-assemble to form empty capsids that are morphologically and antigenically similar to native B19 virus and are thus particularly suited to the study of antibody response against conformational epitopes by ELISA and immunofluorescence assays. B19 structural proteins expressed in bacterial systems, however, have the advantage of being produced in large amounts but, losing the native conformation of B19 virus, are generally suitable for the detection of antibodies directed to B19 linear epitopes, mainly in immunoblot formats.

Recently, Söderlund et al. [1995], using native and denatured VP2 capsid proteins to compare IgG reactivities to conformational and linear epitopes in ELISA and immunoblot formats, found that IgG antibodies against linear VP2 epitopes disappear abruptly within 6 months from the onset of symptoms and are absent in patients with past immunity, while IgG antibodies anti-VP1 linear epitopes and anti-VP2 conformational antigens persist. Therefore, the aim of our work was to produce recombinant VP1 and VP2 proteins and to employ them to verify if the detection of IgG immune response against linear epitopes of VP1 and VP2 can be useful to determine the different phases of B19 infection.

Grant sponsor: CNR Target Project on Biotechnology; Grant sponsor: University of Bologna Funds for Selected Research Topics.

*Correspondence to: Dr. Monica Musiani, Division of Microbiology, Via Massarenti 9, 40138 Bologna, Italy. E-mail: musiani@med.unibo.it

Accepted 13 July 1998

MATERIALS AND METHODS

Samples

A total of 222 serum samples from 218 subjects with different clinical conditions and laboratory evaluations with regards to B19 infection was examined for IgG against VP1 and VP2 linear antigens. All the subjects involved in the study were aged between 3 and 63 years and apparently had no underlying immunological diseases. Immunocompromised patients, such as HIV-infected subjects, patients with leukemia, and transplant recipients, were excluded from the study. All the sera had previously been examined for the presence of B19 DNA using dot blot hybridization and nested PCR, performed as previously described [Zerbini et al., 1990; Musiani et al., 1993] and for the presence of specific IgM and IgG against VP2 conformational epitopes using ELISA commercial preparations (IDEIA, Parvovirus B19, IgM and IDEIA, Parvovirus B19, IgG by Dako A/S, Glostrup, Denmark).

The samples were as follows:

Nine serum samples from nine patients with a B19 acute infection at the onset of symptoms, positive for B19 DNA with or without specific IgM but without IgG against VP2 conformational epitopes by ELISA. This group consisted of six patients with aplastic crisis, one patient with thrombocytopenia and neutropenia, one patient with fever, and one with rash.

Twenty-four serum samples from 24 patients with a symptomatic B19 active/very recent infection diagnosed by the presence of B19 DNA and/or IgM and of IgG against VP2 conformational epitopes by ELISA. This group consisted of 10 patients with rash, 7 with arthritis, 6 with bone marrow hypoplasia, and 1 with glomerulonephritis.

Nine serum samples from five convalescent-phase patients, negative for B19 DNA and IgM but positive for IgG against VP2 conformational epitopes by ELISA. Sera were collected between 3 and 8 months after a documented acute symptomatic B19 infection.

Thirty-eight serum samples sent to our laboratory without a specific request of B19, showing a past B19 infection, i.e., absence of B19 DNA and IgM and presence of IgG against VP2 conformational antigens by ELISA.

Eighty-four sera collected during a nonepidemic period (January–March 1997) from 84 controlled blood donors showing a past B19 infection. The blood donors had proved negative for B19 DNA in the last 3 years, negative at specific IgM but positive for IgG against VP2 conformational epitopes by ELISA.

Forty sera collected during a nonepidemic period (January–March 1997) from 40 healthy blood donors without IgG immune response against VP2 conformational antigens and also negative for B19 DNA and specific IgM.

Eighteen serum samples from 18 pregnant women at the time of clinical presentation of fetal hydrops diagnosed as B19 induced by the presence of B19 DNA in amniotic fluid and/or fetal cord blood [Zerbini et al., 1996]. Of the 18 mothers, 6 were negative and 12 were positive for IgG anti-VP2 conformational antigens by ELISA.

Recombinant VP1 and VP2 Production

The vector used for *E. coli* expression of human parvovirus B19 capsid proteins was constructed by transferring the Hpa I-Sca I fragment from PinPoint Xa-1 Vector (Promega, Madison, WI), containing the tac promoter and a sequence for a fusion biotinylated 13KDa peptide, into Eco 47 III- and Sca I-digested pBR322. The fragments of VP1 and VP2 coding regions (2.3 kb and 1.6 kb, respectively) were obtained by PCR amplification of a plasmid containing the complete internal unique sequence of parvovirus B19 DNA (pB19-A) [Gallinella et. al., 1993]. Amplification primer sequences were designed to obtain *Hind*III restriction sites as a part of the upstream primers (c2630 GGGAAGCTTCAAAAGAAAGTGGCAAATGGTG, c3335 GGGAAGCTTCAACTGGTGCAGGAGGGGGT) and *Not*I restriction sites as a part of the unique downstream primer (c4966 GGGGCGGCCGCTTAGTGATGGTGATGGTGATGCAATGGGTGCACACGGCT). The unique downstream primer was synthesized with a sequence coding for six histidines at the 3′ end to facilitate subsequent purification by metal chelate affinity chromatography. Primers c2630 and c4966 were used for the amplification of the complete VP1 open reading frame; primers c3335 and c4966 were used for the amplification of the VP2 gene.

Polymerase chain reaction was performed by the hot start method using Pwo DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). The DNA was amplified in 40 cycles, each consisting of denaturation for 30 sec at 95°C, annealing for 30 sec at 52°C, and extension for 2 min at 72°C.

All amplification products were cleaved with *Hind*III and *Not*I and ligated to *Hind*III-*Not*I digested vector; the ligation mixtures were used to transform *E. coli* DH5α competent cells and selected colonies were grown and screened to identify clones containing recombinant plasmids with VP1 and VP2 genes. Clones pHV1 and pHV2, respectively, were thus obtained.

Expression of the recombinant proteins was induced by addition of 100 μM IPTG (isopropyl β-D-thiogalactopyranoside) for 5 hr at 37°C and the presence of VP1 and VP2 fusion proteins was tested by SDS-PAGE (8% acrylamide) followed by staining with Comassie Brillant Blue.

Purification of Recombinant Proteins

Cells were lysed in Lysis buffer (Tris-Cl 20 mM pH 8, NaCl 100 mM, GuHCl 6 M, triton 0.1%, imidazole 3 mM), then the cellular lysates were loaded on TALON metal affinity spin columns (Clontech Laboratories, CA). The columns were washed twice with Wash buffer (Tris-Cl 20 mM pH 8, NaCl 100 mM, urea 8 M, imidazole 15 mM) and eluted with Elution Buffer (Tris-Cl 20 mM pH 8, NaCl 100 mM, urea 8 M, imidazole 100 mM). Fractions were tested for the recombinant pro-

TABLE I. B19 Virological and Serological Testings in Serum Samples Taken From Subjects in Different Phases of B19 Infection

Phase of B19 infection	Number of sera examined	Number of serum samples positive			
		B19 DNA/IgM	IgG anti-VP2 conformational epitopes	IgG anti-VP1 linear epitopes	IgG anti-VP2 linear epitopes
Acute	9	9	0	0	0
Active/very recent	24	24	24	24 (100%)	20 (83.3%)
Convalescent (3–8 months after infection)	9	0	9	9 (100%)	9 (100%)
Past infection	38	0	38	29 (76.3%)	8 (21%)
Past infection (blood donors)	84	0	84	59 (70.2%)	16 (19%)
Absence of a documented prior infection (blood donors)	40	0	0	2 (5%)	2 (5%)

tein by SDS-PAGE (8% acrylamide) followed by staining with Comassie Brillant Blue.

Immunoblot

The purified capsid proteins (VP1 and VP2) were loaded in equal amounts and were separated under denaturing conditions by SDS-PAGE (8% acrylamide). They were then transferred [Towbin et al., 1979] to a nitrocellulose membrane and the membrane was cut longitudinally into strips. Each strip was treated with Blocking buffer (1% dried milk in NaCl 150 mM, Tris-Cl 100 mM pH 7.5) for 2 hr and then incubated for 1 hr with human sera (used at the optimal dilution of 1:100 in Blocking buffer). After washing with PBS-Tween 20 0.3%, the strips were incubated for 1 hr with peroxidase-conjugated antihuman IgG (Dako A/S, Glostrup, Denmark) diluted 1:1,000 in Blocking buffer, then developed with 4-chloro-1-naphtol (Bio-Rad Laboratories, Milan, Italy). The optimal working dilution of each immune reagent used in the reaction was determined by preliminary block titration.

Five reference sera from convalescent patients that had previously proved positive by immunoblot for IgG determination against VP1 and VP2 antigens using two commercial assays (Parvoblot Test by Biotrin International, Dublin, Ireland, and Parvovirus B19 Marblot System by Arnika, Milan, Italy) and 10 negative reference sera from nonimmune subjects that had proved negative for IgG determination against VP1 and VP2 antigens at commercial immunoblots were used to assess the specificity and reproducibility of the assay. Two positive and two negative reference sera were included in each group of experiments.

RESULTS

The capsid proteins VP1 and VP2 of human parvovirus B19 were cloned and expressed in *E. coli* as fusion proteins; they were then purified by metal chelate affinity chromatography. SDS-PAGE analysis of purified recombinant proteins showed bands of approximately 97 and 71 KDa, corresponding to the expected size of VP1 and VP2 proteins, respectively, fused with the 13 KDa peptide. Purified proteins were then used by immunoblot assays to detect specific antibodies present in serum samples. A series of control experiments were performed to determine the specificity of our immunoblot assay. First, a positive reaction for IgG against both VP1 and VP2 linear epitopes was observed with the five positive reference sera. Second, a negative reaction was observed with the 10 negative reference sera. Third, no positive reaction was observed when the primary incubation was either omitted or replaced with PBS. Finally, immunoblots were completely unstained when the treatment with peroxidase-conjugated antihuman IgG was omitted or replaced with PBS. To assess the reproducibility of the assay, the 5 positive sera and the 10 reference negative against VP1 and VP2 linear epitopes were retested in 3 different runs and on 3 different days and the expected results were obtained once more.

Having assessed the specificity and reproducibility of the immunoblot assay, we determined the IgG immune response against VP1 and VP2 linear epitopes in the selected groups of samples.

The results obtained from the testing of serum samples taken from subjects in different phases of B19 infection, with the exception of pregnant women with hydropic fetuses, are summarized in Table I.

Out of the nine samples from patients with B19 acute infection, positive for B19 DNA and/or IgM but negative for IgG against VP2 conformational epitopes, none proved positive for IgG against both VP1 and VP2 linear epitopes.

Out of the 24 samples from patients with an active/very recent symptomatic B19 infection, positive for B19 DNA and/or IgM and already positive for IgG against VP2 conformational epitopes, all (100%) proved positive for IgG against VP1 linear antigens and 20 (83.3%) proved positive for IgG anti-VP2 linear epitopes. Out of the nine sera from five convalescent–phase patients, all (100%) had IgG both for VP1 and for VP2 linear epitopes.

Out of the 38 serum samples showing signs of a past B19 infection, 29 (76.3%) proved positive for IgG anti-VP1 linear antigens and 8 sera (21%) proved positive for IgG anti-VP2 linear antigens. Of the eight samples positive at IgG anti-VP2 linear antigens, all were positive for IgG anti-VP1 linear antigens.

Out of the 84 serum samples from healthy blood donors showing signs of past B19 infection, 59 (70.2%) were positive for IgG against VP1 linear antigens and 16 (19%) sera were positive for IgG against VP2 linear

antigens. The 16 serum samples positive for IgG against VP2 linear antigens were also positive for IgG anti-VP1 linear epitopes.

Out of the 40 sera from controlled blood donors without a preexisting immunity against VP2 conformational epitopes, 38 (95%) were negative for IgG against both VP1 and VP2 linear antigens while 2 samples (5%) were positive for both IgG anti-VP1 and -VP2 linear epitopes.

When the group of 18 serum samples from 18 pregnant women collected at the time of clinical presentation of B19-induced fetal hydrops were analyzed, six mothers who were negative for IgG against VP2 conformational epitopes were also negative for IgG against VP1 and VP2 linear epitopes.

Out of the 12 samples from mothers who had already mounted an IgG immunity against VP2 conformational epitopes, 9 (75%) sera were positive and 3 (25%) were negative at IgG against VP1 linear epitopes, while 4 (33%) were positive and 8 (67%) were negative at IgG against VP2 linear epitopes.

DISCUSSION

The IgG immune response against VP1 and VP2 linear epitopes was determined by immunoblot in 222 serum samples from 218 different subjects in different phases of B19 infections excluding immunocompromised patients where an altered or even absent B19-specific immune response has often been documented.

The IgG immune response against VP1 and VP2 linear epitopes was studied by immunoblots in which equal amounts of the two proteins were present on the same strip in order to avoid an unbalanced presence of the two antigens deriving from the preparation of immunoblots with native B19 virus where the two antigens (VP1 and VP2) are present with a ratio 1:19.

In the active or very recent phase of infection, IgG against VP1 linear antigens were detectable concomitantly and with the same frequency as IgG against VP2 conformational epitopes, while in convalescent sera also IgG against VP2 linear epitopes were detectable with the same frequency as IgG against VP2 conformational antigens. Previous studies using immunoblot assays [Schwartz et al., 1988; Kurtzman et al., 1989] have suggested that IgG anti-VP2 linear antigens are detectable prior to IgG anti-VP1 linear antigens, but the lack of detection of early IgG anti-VP1 might be due to the fact that in those studies immunoblots were prepared using native virus as source of antigens, thus with about a 19-fold higher amount of VP2 antigen with respect to VP1 on each strip.

In past infection sera, the antibody response against VP1 linear epitopes together with antibody response to VP2 conformational epitopes was seen more persistent than IgG response against VP2 linear epitopes. These findings correlate with a protective role of antibody against VP1 linear and VP2 conformational epitopes since most of neutralizing epitopes in the VP1 region appear to be linear in contrast to those in VP2, which are prevalently conformational [Kajigaya and Momoeda, 1997]. Our results, which show that IgG presence against VP1 linear epitopes is more dominant than IgG response against VP2 linear epitopes in past infection sera, are in accordance with previous data [Kurtzman et al., 1989; Söderlund et al., 1995; Palmer et al., 1996], which showed that VP1 is the major antigen recognized by past infection sera and by commercial immunoglobulins in immunoblot assays. The fact that IgG anti-VP1 linear epitopes were detectable in 76.3% and 70.2% of subjects of the two groups with past infection compared to 100% of subjects with recent infection may be due to the fact that after the convalescent phase the quantity of specific IgG decreases and cannot reach the sensitivity limit of immunoblot assay.

The data regarding the presence of IgG anti-VP2 linear antigens in about 20% of subjects with a past B19 infection showed that the detection of IgG anti-VP2 linear epitopes can be considered a marker of a relative recent infection in most cases but does not seem totally suitable as a single probatory test to detect an active B19 infection. The data, therefore, were only partially consistent with the results of Söderlund et al. [1995], which showed a strict correlation between the presence of IgG anti-VP2 linear epitopes and active B19 infection, with an abrupt disappearance of IgG against VP2 linear epitopes within 6 months after infection.

The group of pregnant women with serum samples taken at the time of clinical presentation of B19 fetal hydrops could be considered in the active/very recent or convalescent phase of infection since fetal hydrops can occur 1–12 weeks after maternal symptomatic or asymptomatic infection. The 12 mothers who had mounted an IgG immune response against VP2 conformational antigens, however, showed a lower IgG immune response against VP1 and VP2 linear epitopes than that observed in the other groups of patients studied in the active/very recent or convalescent phases of infection. Whether the partial lack of IgG immune response against VP1 and VP2 linear epitopes in pregnant women can reflect a partially impaired immunoreactivity that can favor a symptomatic infection of the fetus needs to be verified in a higher number of cases.

In 95% of the serum samples collected from blood donors without IgG against VP2 conformational epitopes, the negativity was associated with the lack of IgG against VP1 and VP2 linear antigens but in 5% a positivity was found for IgG against both VP1 and VP2 linear antigens. Since the latter data were confirmed positive by using two different commercial immunoblots, these results would suggest that these sera reflected a prior contact with B19 and that in some cases, albeit rarely, there may be a presence of IgG against linear epitopes in the absence of IgG against VP2 conformational antigens.

In the analysis of the total 222 screened sera, all the samples found positive for an IgG immune response against VP2 linear antigens were also positive for IgG against VP1 linear antigens. These results had been expected since VP1 and VP2 proteins, derived from overlapping reading frames, are identical except that

VP1 contains a unique portion of 227 additional amino acids at the amino terminus. Therefore, serum samples with IgG against VP1 linear antigens but lacking IgG against VP2 linear antigens should be considered positive for IgG against VP1 linear epitopes exclusively in the VP1 unique region. This is particularly important for the sera collected from subjects with a past contact with B19 where the presence of IgG against VP1 unique region linear epitopes lacking IgG anti-VP2 linear epitopes seems a common feature. Since the VP1 unique region is situated externally on the capsid and contains multiple neutralizing linear epitopes, it appears that IgG against this portion may have an enduring protective role.

In conclusion, the determination of IgG anti-VP1 and -VP2 linear epitopes in different groups of subjects along with the determination of IgG against VP2 conformational epitopes has demonstrated that conformational and linear epitopes elicit a differential IgG immune response during the course of B19 infection. Moreover, the data demonstrate that the immunoblot analysis of IgG response against VP1 and VP2 linear epitopes can be useful to outline a more defined profile of the immune status against B19.

REFERENCES

Anderson LJ. 1990. Human parvoviruses. J Inf Dis 161:603–608.

Brown CS, Salimans MMM, Noteborn MHM, Weiland HT. 1990. Antigenic parvovirus B19 coat proteins VP1 and VP2 produced in large quantities in a baculovirus expression system. Virus Res 15:197–212.

Brown KE, Young NS, Liu JM. 1994. Molecular, cellular and clinical aspects of parvovirus B19 infection. Crit Rev Oncol Hematol 16: 1–31.

Gallinella G, Musiani M, Zerbini M, Gentilomi G, Gibellini D, Venturoli S, La Placa M. 1993. Efficient parvovirus B19 DNA purification and molecular cloning. J Virol Methods 41:203–212.

Kajigaya S, Fujii H, Field A, Anderson S, Rosenfeld S, Anderson LJ, Shimada T, Young NS. 1991. Self assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. Proc Natl Acad Sci USA 88:4646–4650.

Kajigaya S, Momoeda M. 1997. Immune response to B19 infection. In Anderson LJ, Young NS, editors. Human parvovirus B19. Basel: Karger, p 120–136.

Kurtzman GJ, Cohen B, Field AM, Oseas R, Blaese RM, Young NS. 1989. Immune response to B19 parvovirus and an antibody defect in persistent viral infection. J Clin Invest 84:1114–1123.

Morinet F, D'Auriol L, Tratschin JD, Galibert F. 1989. Expression of the human parvovirus B19 protein fused to protein A in *Escherichia coli:* Recognition by IgM and IgG antibodies in human sera. J Gen Virol 70:3091–3097.

Musiani M, Azzi A, Zerbini M., Gibellini D, Venturoli S, Zakrzewska K, Re MC, Gentilomi G, Gallinella G, La Placa M. 1993. Nested polymerase chain reaction assay for the detection of B19 Parvovirus DNA in human immunodeficiency virus patients. J Med Virol 40:157–160.

Palmer P, Pallier C, Leruez-Ville M, Deplanche M, Morinet F. 1996. Antibody response to human parvovirus B19 in patients with primary infection by immunoblot assay with recombinant proteins. Clin Diagn Lab Immunol 3:236–238.

Pattison JR. 1994. Human parvovirus B19. Lancet 308:149–150.

Schwartz TF, Roggendorf M, Deinhardt F. 1988. Human parvovirus B19, ELISA and immunoblot assay. J Virol Methods 20:155–168.

Söderlund M, Brown KE, Meurman O, Hedman K. 1992. Prokariotic expression of a VP1 polypeptide antigen for diagnosis by a human parvovirus B19 antibody enzyme immunoassay. J Clin Microbiol 30:305–311.

Söderlund M, Brown CS, Spaan WJM, Hedman L, Hedman K. 1995. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Inf Dis 172:1431–1436.

Towbin H, Staehelin T, Gordon G. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitro cellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Zerbini M, Musiani M, Venturoli S, Gallinella G, Gibellini D, Gentilomi G, La Placa M. 1990. Rapid screening for B19 parvovirus DNA in clinical specimens with a digoxigenin-labeled DNA hybridization probe. J Clin Microbiol 28:2496–2499.

Zerbini M, Musiani M, Gentilomi G, Venturoli S, Gallinella G, Morandi R. 1996. Comparative evaluation of virological and serological methods in prenatal diagnosis of parvovirus B19 fetal hydrops. J Clin Microbiol 34:603–608.